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Full Length Research Paper

Antioxidant and antihepatotoxic activities of ethanolic crude extract of *Melia azedarach* and *Piper longum*

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In the present investigation, the antioxidant and antihepatotoxic activities of the crude ethanolic extract of *Melia azedarach* and *Piper longum* were studied. The antioxidative activities of biherbal ethanolic extract (BHEE) were investigated employing various established *in vitro* systems such as total antioxidant activity in linoleic acid emulsion system and inhibitory effect on protein oxidation. Total phenolic and flavonoid content of the extract (BHEE) were also determined by a colorimetric method. The biherbal ethanolic extract also showed potent antihepatoxic activity against carbon tetrachloride – induced acute toxicity in rat liver. The BHEE at a dose level of 50 mg/kg body weight were administered to different groups of rats orally once daily for 14 days. The degree of liver protection was determined by estimating the levels of serum marker enzymes such as SGOT, SGPT, ALP, ACP and LDH. Silymarin at a dose level of 50 mg/kg was used as standard. The results revealed that BHEE has notable inhibitory activity on peroxides formation in linoleic acid emulsion system in a dose-dependent manner. Moreover, free radical-induced protein oxidation was suppressed significantly by the addition of BHEE over a range of concentration, as revealed by the marked elevation of serum marker enzyme levels in CCl_4 treated rats. The amelioration of liver toxicity by BHEE was evident from its significant effect on the levels of serum marker enzymes such as SGOT, SGPT, ALP, ACP and LDH. The results of this study strongly indicate that the hepatoprotective effect of the BHEE is possibly related to its marked antioxidant activity.

Key words: Hepatoprotective, marker enzymes, Biherbal ethanolic extract (BHEE), carbon tetra chloride.

INTRODUCTION

Reactive oxygen species (ROS) are generated by many redox processes that normally occur in the metabolism of aerobic cells. ROS include free radicals such as super oxide (O_2^-), hydroxyl radical ($^{\cdot}OH$), peroxyl radical (RO^{-2}) as well as non-radical species such as hydrogen peroxide (H_2O_2) (Cerutti, 1991). These species are highly reactive and harmful to the cells. If not eliminated, ROS can damage important molecules, such as proteins, DNA and lipids. Cells express several defense mechanisms, including antioxidant enzymes and nonenzymatic compounds, that help prevent the damaging effects of ROS (Ben-Yoseph et al., 1996; Fridovich, 1997). However, these endogenous systems are often insufficient for complete scavenging of ROS. In aerobic organisms, one of the major targets of ROS is the cellular biomembranes where they induce lipid peroxidation. Lipid peroxidation not only affects the membrane structure and its function but also generate some oxidation reaction products. These oxidation products can react with biomolecules and exert cytotoxic and genotoxic effect.

High levels of lipid peroxides have been found in the

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Abbreviations: BHEE, Biherbal ethanolic extract; **CCl_4**, carbon tetrachloride; **ALP**, alkaline phosphatase; **ACP**, acid phosphatase; **LDH**, lactate dehydrogenase; **BHT**, butylated hydroxytoluene; **SGOT**, serum glutamate oxaloacetate transaminase; **SGPT**, glutamate pyruvate transaminase; **BSA**, bovine serum albumin; DNPH, 2,4-dinitrophenylhydrazine; **CYP2E1**, cytochrome P450 2E1; **PCO**, protein carbonyl formation.

serum of patients suffering from liver disease, diabetes, vascular disorders and tumors (Pezzuto and Park, 2002). Oxidative stress can also play an important role in the development of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Behl and Mosmann, 2002).

There is an increasing interest in natural antioxidants, namely phenols, present in medicinal and dietary plants, that might help prevent oxidative damage (Gardner et al., 2000; Youdim et al., 2002). The administration of an antioxidant source comprising of multiple components could offer protection against cancer and combat oxidative stress –induced physiological malfunctions (Ningappa et al., 2007). In situations of increased free radical generation, the reinforcement of endogenous antioxidants via intake of dietary antioxidants may be of particular importance in attenuating the cumulative effects of oxidatively damaged molecules.

With the above scenario, the biherbal extract (BHEE), made up equal quantities of leaves of *Melia azedarach* and seeds of *Piper longum,* were subjected to various assays in order to evaluate their antioxidant and antihepatotoxic activities. *Melia azedarach*, a member of the family Meliaceae is widely grown as an ornamental tree, being used against intestinal worms, in skin diseases, stomach ache, intestinal disorders, uterine illnesses, cystitis, diuretic and febrifuge (Perry, 1980). It has got antiviral, antimalarial, anti-helmintic and cytotoxic activeties (Castilla et al., 1998). *Piper longum,* an important medicinal plant belonging to the family Piperaceae, is being used in traditional medicine by many people in Asia and Pacific islands especially in Indian medicine (Guido et al., 1998). *Piper longum* is a component of medicines reported as good remedy for treating gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain and arthritic conditions (Singh, 1992). In this study, we therefore assessed the combined antioxidant and hepatoprotective activity from the crude extracts of these two plants.

MATERIALS AND METHODS

Plant material

The leaves of *M. azedarach* and seeds of *P. longum* were collected from the center for Advanced Studies in Botany Field Research Laboratory, University of Madras, Chennai, India and were authenticated by Dr. P.T Kalaichelvan (Professor, Advanced Studies in Botany, University of Madras, Chennai, India). The voucher specimen is available in the herbarium file of the Studies in Botany Field Research Laboratory, University of Madras, Chennai, India.

Extraction

The leaves of *M. azedarach* (1 Kg) and seeds of *P. longum* (1 Kg) were shade-dried and pulverized to a coarse powder. Equal quantities of the powder was passed through 40-mesh sieve and exhaustively extracted with 90% (v/v) ethanol in soxhlet apparatus at 60°C. The extract was evaporated under pressure until all the solvent had been removed and further removal of water was carried out by freeze drying to give an extract sample with the yield of 19.7% (w/w). The extract was stored in refrigerator for further use.

Animals

Adult albino male rats of wistar strain weighing 150 - 175 g were used in the pharmacological and toxicological studies. The inbred animals were taken from animal house in Madras Medical College, Chennai, India. The animals were maintained in well-ventilated room temperature with natural 12 ± 1 h day–night cycle in the propylene cages. They were fed balanced rodent pellet diet from Poultry Research Station Nandam, Chennai, India and tap water *ad libitum* was provided throughout the experimental period. The animals were sheltered for one week and prior to the experiment they were acclimatized to laboratory temperature. The protocol was approved by Animal Ethics Committee constituted for the purpose as per CPCSEA Guideline.

Assay of antioxidant activities

Total antioxidant activity

The total antioxidant activity of BHEE was measured by use of a linoleic acid system by the method of Mitsuda et al. (1996). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 emulsifier and 50 ml of phosphate buffer (0.2 M, pH 7.0). The mixture was then homogenized. A 0.5 ml of different concentration of the extract and standard sample (in ethanol) was mixed with linoleic acid emulsion (2.5 ml, 0.2 M, pH 7.0) and phosphate buffer (2 ml, 0.2 M, pH 7.0). The reaction mixture was incubated at 37°C in the dark to accelerate the peroxidation process. The levels of peroxidation were determined according to the thiocyanate method by sequentially adding ethanol (5 ml, 75%), ammonium thiocyanate (0.1 ml, 30%), sample solution (0.1 ml) and ferrous chloride (0.1 ml, 20 mM $FeCl_2$ in 3.5% HCl). Butylated hydroxytoluene (BHT) was used as positive control. After mixing for 3 min, the peroxide values were determined by reading the absorbance at 500 nm.

Determination of total phenolic content

Total phenolic content in the lyophilized extract was determined with the Folin–Ciocalteu's reagent (FCR) according to a published method of Slinkard and Singleton (1977). 100 mg of the sample dissolved in 0.5 ml of water was mixed with 2.5 ml FCR (diluted 1:10, v/v) followed by 2 ml of Na_2CO_3 (7.5%, v/v) solution. The absorbance was then measured at 765 nm after incubation at 30°C for 90 min. Results were expressed as gallic acid equivalents (mg gallic acid/g dried extract).

Determination of total flavonoid content

The total flavonoid content of BHEE was determined by a colorimetric method as described in the literature of Zhishen et al. (1999). The 100 mg sample (0.5 ml) was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a $NaNO_2$ solution (15%). After 6 min, 0.15 ml of an $AlCl_3$ solution (10%) was added and allowed to stand for 6 min, then 2 ml of NaOH solution (4%) was added to the mixture. Immediately, water was added to bring the final volume to 5 ml and the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was then determined at 510 nm versus prepared water blank. Results were expressed as catechin equivalents (mg catechin/g dried extract).

Assay of protein oxidation

The effects of BHEE on protein oxidation were carried out according to the slightly modified method of Wang et al. (2006). Bovine serum albumin (BSA) was oxidized by a Fenton-type reaction. The reaction mixture (1.2 ml), containing sample extract (100 – 1000 μg/ml), potassium phosphate buffer (20 mM, pH 7.4), BSA (4 mg/ml), $FeCl_3$ (50 μM), H_2O_2 (1 mM) and ascorbic acid (100 μM) was incubated for 30 min at 37^0C. For determination of protein carbonyl content in the samples, 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added to the reaction mixture. Samples were incubated for 30 min at room temperature. Then, 1 ml of cold TCA (10%, w/v) was added to the mixture and centrifuged at 3000 g for 10 min. The protein pellet was washed three times with 2 ml of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 ml of guanidine hydrocholoride (6 M, pH 2.3). The absorbance of the sample was read at 370 nm. The data were expressed in terms of percentage inhibition, calculated from a control measurement of the reaction mixture without the test sample.

Assay of hepatoprotective activity

Carbontetra chloride induced hepatotoxicity in rats was used as a model to determine the hepatoprotective activity of the BHE. The rats were divided into five groups with six animals in each group and were given dose schedule as: Group I: Animals were given a single administration of 0.5 ml vehicle (2% (v/v) aqueous Tween 80) p.o for 14 days. This group served as control; Group II, III, and V: Animals were given a single dose of 2 ml/kg, p.o CCl_4 (2% (v/v) aqueous Tween 80) daily for 7 days; Group III: Animals were treated with 50 mg/kg, p.o of (BHE) daily for 14 days; Group IV: Animals received only 50 mg/kg, p.o of BHE) daily for 14 days; Group V: Animals received 50 mg /kg p.o Silymarin in 2% (v/v) aqueous Tween –80 daily for 14 days. This group served as positive control. On the 15th day the animals were sacrificed and various biochemical parameters were analyzed.

At the end of the experimental period animals were sacrificed by cervical decapitation under mild pentobarbitone anesthesia, blood was collected and the serum was separated by centrifuging at 3,000 rpm for 10 min. The above collected serum was used for the assay of marker enzymes. The enzymes glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were determined by the method of Reitman and Frankel. Alkaline phosphatase (ALP) acid phosphatase (ACP) were determined by the method of Kind PRN and King EJ. The enzyme lactate dehydrogenase (LDH) was determined by the method of King J.

Statistical analysis

Values reported are the mean ± S.E.M. The statistical comparisons were done using one way analysis of variance (ANOVA) followed by Dunnet's't' test. P values < 0.05 were considered as significant.

RESULTS

Assay of antioxidant activities

Total antioxidant activity

The total antioxidant activity of BHEE was measured using ferric thiocyanate test which determines the amount of peroxide produced at the initial stage of lipid peroxidation. Lower absorbance indicates a higher level of antioxidant activity. Figure 1 shows the changes in the absorbance under the influence of different concentrations of the extract (100 – 1000 μgm/ml) at 37°C compared to BHT as a positive control. According to this Figure the extent of inhibition of lipid oxidation is moderate at low (100 μg/ml) doses of BHEE. However, at higher concentrations (800 and 1000 μg/ml), BHEE suppressed lipid oxidation to a considerable extent.

Total phenolic and flavonoid contents

The antioxidant activity of BHEE is probably due to its phenolic contents. It is well known that phenolic com--pounds are constituents of many plants and they have attracted a great deal of public and scientific interest because of their health promoting effects as antioxidants. Flavonoids are a class of secondary plant phenolics with powerful antioxidant properties. Therefore, it would be valuable to determine the total phenolic and flavonoid content of the plant extracts. The extracts were investigated regarding their composition by different colorimetric techniques, such as the content of total phenolic compounds by the Folin– Ciocalteu's assay and flavonoids by AlCl3 reagent. Table 1 show the total phenolic and flavonoid contents of BHEE were determined and expressed in terms of gallic acid and catechin equivalents. Total phenolic and Flavonoid contents of each gram of dried extract were estimated to be equivalent to 74.8 mg gallic acid and 49.8 mg catechin. The antioxidant activity of BHEE is probably due to its phenolic content and the secondary plant phenolics the flavonoids.

Inhibitory effects against protein oxidation

The oxidative protein damages, provoked by free radicals, have been demonstrated to play a significant role in aging and several pathological events. Radical mediated damages to proteins might be initiated by electron leakage, metal-ion dependent reactions and auto-oxidation of lipids and sugars. Major molecular mechanisms, leading to structural changes in proteins are free-radical mediated protein oxidation characterized by carbonyl formation (PCO). The protein oxidation was determined in terms of inhibition protein carbonyl formation (PCO). As shown in Figure 2 BHEE dose- dependently exhibited inhibitory effects of PCO formation by 22.46, 41.34, 55.24, 64.93, 70.93 and 78.94% at the extract concentration of 100, 200,400, 600, 800 and 1000 μgm/ml, respectively.

CCl_4-induced hepatotoxicity

The activities of SGOT, SGPT, ALP, ACP and LDH after intoxication of single dose of 2 ml/kg, p.o CCl_4 (2% (v/v)

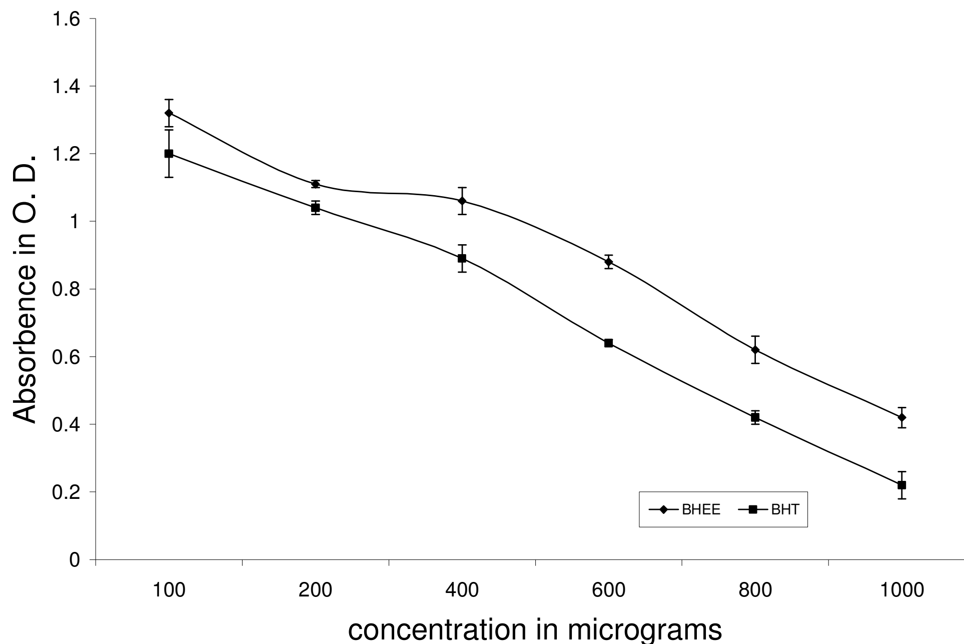

Figure 1. Total antioxidant activity of different concentrations of bi-herbal ethnolic extract (BHEE) and BHT in linoleic acid emulsion determined by thiocyanate method Each value represent mean ± SEM (n = 3).

Table 1. Total Phenolic and flavonoid content of Biherbal ethanolic extract.

Total phenolic content (mg/g)	74.8 ± 1.24
Total flavonoid content (mg/g)	49.8 ± 1.48

Each value represents the mean ± SEM (n = 3).
Total phenolic content was expressed as mg gallic acid equivalents/g dried extract.
Total flavonoid content was expressed as mg catechin equivalent/g dried extract.

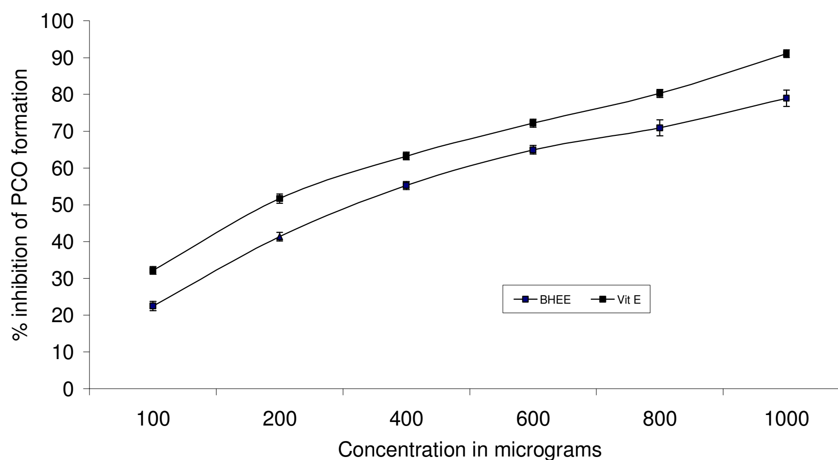

Figure 2. Inhibitory effect of bi-herbal ethanolic extract (BHEE) and vitamin E on protein oxidation expressed as protein carbonyl formation (PCO) induced by $H_2 O_2$ /Fe^{2+}/ ascorbic acid system. Each value represent mean ± SEM (n = 3).

Table 2. Marker enzymes.

Enzyme	Group I	Group II	Group III	Group IV	Group V
SGOT (U/L)	46.5 ± 1.1	143.79 ± 4.5a*	87.30 ± 3.4b*	38.75 ±1.46b*	76.92± 3.6c^{NS}
SGPT (U/L)	46.00 ± 1.03	145.50±1.08a*	75± 0.98b*	45.50 ± 1.66b*	78.16±0.54c^{NS}
ALP (K.A)	76.66 ± 0.53	172.68±0.64a*	121.75±0.72.b*	76.16±0.38b*	121.28±1.0c^{NS}
ACP (K.A)	4.11 ± 0.05	12.2± 1.06a*	6.76 ±0.24.b*	3.2 ± 0.15b*	6.7 ±0 .29c^{NS}
LDH (U/L)	145.9 ± 1.87	435.38±1.84a*	253.0 ±1.50.b*	135.26±0.87b*	240.7± 2.9c^{NS}

Values are Mean ± SEM of 6 animals each in a group. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's `t' test (n = 6); Comparison between: a–Group I vs. Group II, b–Group II vs. Group III and IV, c–Group I vs. Group V. *P < 0.001, NS–Not Significant.

aqueous Tween 80) daily for 7 days, are summarized in Table 2. The results indicated that the marker enzyme levels were increased significantly ($p < 0.001$) in-group II animals but were brought back to the near normal levels in Group III (BHEE treated) animals. These animals show a significant decrease in marker enzymes ($p < 0.001$). Oral administration of BHEE more significantly decreased the levels of SGOT, SGPT, ACP ALP and LDH when compared to those of CCl_4 treated Group II animals. All the parameters were under normal limits in the silymarin treated group, which acted as a positive control. Comparisons between Group I, Group II and IV shows no significant variation in marker enzyme levels indicating no appreciable adverse side effects due to the administration of Tween – 80 and BHEE alone in Group I and Group V animals.

DISCUSSION

Lipid oxidation is one of the major factors causing deterioration of foods during the storage and processing. Oxidized polyunsaturated fatty acids may induce aging and carcinogenesis. Although there are some synthetic antioxidant compounds such as BHT and butylated hydroxyanisole (BHA), which are commonly used in foods processing, it has been reported that these synthetic antioxidants are not devoid of biological side effects and their consumption may lead to carcinogenicity and causes liver damages (Linderschmidt et al., 1986). Therefore, the development of alternative antioxidants mainly from natural sources has attracted considerable attention. The BHEE at higher concentrations inhibits the lipid oxidation indicating, it has considerable quantities of phenols and flavonoids responsible for the antioxidant activity. The phenolic compounds exhibit considerable free radical scavenging activities, through their reactivity as hydrogen- or electron-donating agents and metal ion chelating properties (Rice-Evans et al., 1996)

The oxidative protein damages, provoked by free radicals, have been demonstrated to play a significant role in aging and several pathological events (Reznick and Packer, 1994). Protein oxidation was used as another method to measure hydroxyl radical scavenging activity of BHEE by incubating BSA in a H_2O_2/Fe^{3+}/ascorbic acid system which generate hydroxyl radicals. Protein oxidation was measured in terms of Protein carbonyl formation. In deed, measurement of PCO has been used as a sensitive assay for oxidative damages of proteins. BHEE dose-dependently exhibited inhibitory effects of PCO formation. The inhibitory activity of BHEE is probably due to its antioxidant contents. Substances termed antioxidants can influence the oxidation process through simple or complex mechanisms including prevention of chain initiation, binding of transitional metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging (Ames et al., 1993).

It is well established that CCl_4 induces hepatotoxicity by metabolic activation; therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function. CCl_4 is bio-transformed by the cytochrome *P*450 system (CYP2E1) in the endoplasmic reticulum to produce trichloromethyl free radical ($\bullet CCl_3$). Trichloromethyl free radical when combined with cellular lipids and proteins in the presence of oxygen form trichloromethyl peroxyl radical ($\bullet OOCCl_3$), which may attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical. Thus, trichloromethylperoxyl free radical leads to elicit lipid peroxidation, the destruction of Ca^{2+} homeostasis and finally, results in cell death (Reckengel, 1989). Assessment of liver damage can be made by estimating the activities of serum GOT, GPT, ALP, ACP and LDH, which are enzymes originally present in higher concentration in cytoplasm. When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage. The elevated level of these entire marker enzymes observed in the CCl_4 treated group II rats in this present study corresponded to the extensive liver damage induced by toxin. The tendency of these marker enzymes to return towards a near-normalcy in group III BHEE treated rats was a clear manifestation of anti- hepatotoxic effect of BHEE.

Conclusion

The ethanolic bi-herbal extract exhibited significant hepa-

toprotective effect against CCl_4 induced acute hepatotoxicity in rats. This effect is probably mediated through its significant antioxidant activity.

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